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Endothelial Nitric Oxide Synthase Overexpression Provides a Functionally Relevant Angiogenic Switch in Hibernating Pig Myocardium

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Objectives We investigated whether retroinfusion of liposomal endothelial nitric oxide synthase (eNOS) S1177D complementary deoxyribonucleic acid (cDNA) would affect neovascularization and function of the ischemic myocardium.

Background Recently, we demonstrated the feasibility of liposomal eNOS cDNA transfection via retroinfusion in a model of acute myocardial ischemia/reperfusion. In the present study, we used this approach to target a phosphomimetic eNOS construct (eNOS S1177D) into chronic ischemic myocardium in a pig model of hibernation.

Methods Pigs (n = 6/group) were subjected to percutaneous implantation of a reduction stent graft into the left anterior descending artery (LAD), inducing total occlusion within 28 days. At day 28, retroinfusion of saline solution containing liposomal green fluorescent protein or eNOS S1177D cDNA (1.5 mg/animal, 2 × 10 min) was performed. Furthermore, L-nitroarginine-methylester (L-NAME) was applied orally from day 28, where indicated. At day 28 and day 49, fluorescent microspheres were injected into the left atrium for perfusion analysis. Regional functional reserve (at atrial pacing 140/min) was assessed at day 49 by subendocardial segment shortening (SES) (sonomicrometry, percent of ramus circumflexus region).

Results The eNOS S1177D overexpression increased endothelial cell proliferation as well as capillary and collateral growth at day 49. Concomitantly, eNOS S1177D overexpression enhanced regional myocardial perfusion from 62 ± 4% (control) to 77 ± 3% of circumflex coronary artery–perfused myocardium, unless L-NAME was coapplied (69 ± 5%). Similarly, eNOS S1177D cDNA improved functional reserve of the LAD (33 ± 5% vs. 7 ± 3% of circumflex coronary artery–perfused myocardium), except for L-NAME coapplication (13 ± 6%).

Conclusions Retroinfusion of eNOS S1177D cDNA induces neovascularization via endothelial cell proliferation and collateral growth. The resulting gain of perfusion enables an improved functional reserve of the hibernating myocardium.
Foundation

A growing population of patients who have exhausted current interventional and surgical approaches to treat coronary malperfusion depend on novel therapeutic approaches. One strategy potentially meeting the needs of the "no option" patient consists of therapeutic neovascularization via growth factors such as the vascular endothelial growth factor (VEGF), fibroblast growth factor, insulin-like growth factor (IGF), angiopoietin families among others. Earlier clinical studies using growth factor treatment of the ischemic region yielded mixed results, with singular studies improving the record by using advanced regional delivery (1,2) and vector (3) systems. The concept of therapeutic neovascularization requires angiogenesis (4), defined as capillary sprouting, as well as arteriogenesis (5,6) (e.g., enlargement of preexisting conductance vessels for improvement of tissue perfusion). Recent studies found a contribution of vasculogenesis (7,8)—using endothelial progenitor cells—to vessel growth in the adult organism.

Of note, endothelial nitric oxide synthase (eNOS) is a central enzyme mediating therapeutic neovascularization. In addition to endothelial sprouting, arteriogenesis is impaired in transgenic mice lacking physiological eNOS activation

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Abbreviations and Acronyms

ANOVA = analysis of variance

cDNA = complementary deoxyribonucleic acid

cGMP = cyclic guanosine monophosphate

eGFP = endothelial green fluorescent protein

eNOS = endothelial nitric oxide synthase

IGF = insulin-like growth factor

LAD = left anterior descending artery

L-NAME = L-nitroarginine-methylester

RCx = ramus circumflexus

SES = subendocardial segment shortening

VEGF = vascular endothelial growth factor

(9), whereas eNOS overexpression enhances angiogenesis (10). Mice lacking eNOS expression also display impaired vasculogenic responses (11). Moreover, growth factors of the VEGF (12,13) or IGF family (14) as well as statins (15), all capable of increasing perfusion in ischemic muscle, invariably activate AKT, which phosphorylates eNOS at a critical serine residue, S1177 (12,16). This phosphorylation step is bypassed in a mutant form of eNOS that carries a phosphomimetic amino acid (aspartate) in place of serine residue 1177 (eNOS S1177D), increasing nitric oxide formation significantly. Thus, regional transfection of ischemic muscle tissue with eNOS S1177D might offer an attractive option of therapeutic neovascularization, although this approach has not been assessed in larger animals or preclinical settings.

Therefore, we studied the potential of a constitutively active mutant (eNOS S1177D) to improve performance and perfusion of hibernating myocardium in a preclinical pig model. Chronic ischemia of the left anterior descending artery (LAD) perfusion area was percutaneously induced by implantation of a reduction stent (17). With selective, pressure-regulated retroinfusion, which proved efficient in previous studies in pigs (18), we applied liposomes containing eNOS S1177D regionally to the myocardium 28 days after induction of ischemia, a time point at which hibernating of the myocardium has evolved (17). Evaluation of myocardial perfusion and function was obtained 3 weeks later, to allow for stable neovascularization.

Methods

Materials. All chemicals were purchased from SIGMA (Taufkirchen, Germany). Contrast agent Solutrast 370 was kindly provided by Byk Gulden (Konstanz, Germany). For transfection of eNOS S1177D, Effectene (Qiagen, Hilden, Germany) was used according to the manufacturer's instruction.

Cell adhesion and proliferation in vitro. Rat neonatal coronary endothelial cells (NCECs) were cultured as previously described (19). Proliferation of coronary endothelial cells without or with transfection of eNOS S1177D (with Effectene) was assessed by Ki67 staining (Novocastra, Newcastle upon Tyne, United Kingdom).

Animal instrumentation. All experimental procedures were approved by the Bavarian Animal Care and Use Committee. All pig experiments were conducted at the Institute for Surgical Research of the University of Munich. German farm pigs, pretreated with clopidogrel (300 mg) and aspirin (300 mg), were anesthetized and instrumented as previously described (17). The external jugular vein and the carotid artery of the right side were cannulated and appropriate sheaths (8-F) were placed.

Ischemia. A coronary polytetrafluoroethylene-covered stent-graft (length 13 mm; Jomed, Germany) was ligated, as described previously (17). Thereafter, the stent was crimped on a percutaneous transluminal angioplasty-balloon (3.0 × 20 mm) and implanted into the proximal LAD. The stent did not expand at the site of the ligation, resulting in an hour-glass-shaped stenosis of 75% of the luminal diameter. Maintenance of flow in the LAD was monitored invasively by injection of contrast agent under fluoroscopy at day 7 and day 28 (Fig. 1). Of the 40 animals initiated by stent placement, 11 died during the first 28 days (n = 7 in between the first week), whereas only 1 animal was lost between day 28 and day 49. Five animals were excluded because of early stent occlusion (between day 0 and day 7), inducing myocardial infarction >8% of the left ventricular mass. Of the remaining 24 animals, 18 (n = 6/group) were divided into mock transfection enhanced green fluorescent protein (eGFP), eNOS S1177D, or eNOS S1177D + L-NAME treatment (see the following text), whereas 6 animals were transfected with eGFP or eNOS S1177D (n = 3 each) at day 7 and killed at day 10 for analysis of eNOS S1177D transfection (Fig. 2). According to the inclusion criteria, infarct size (triphenyl-tetrazolium [TTC]-staining at day 49) was constant across all experimental groups (5.6 ± 2.1% in control subjects, 4.1 ± 1.1% in eNOS S1177D, and 4.7 ± 1.8% in eNOS S1177D+L-NAME group).

Retroinfusion. At day 28, after instrumentation (see previous), the retroinfusion catheter was advanced into the anterior interventricular vein (AIV) draining the LAD perfusion area, as described in detail previously (18). After assessment of the individual systolic occlusion pressure of the venous system, retroinfusion pressure was set 20 mm Hg above the latter. Continuous pressure-regulated retroinfusion of isothermic sodium chloride 0.9% (20 ml/min) with liposomes containing either eGFP complementary deoxyribonucleic acid (cDNA) (control) or eNOS S1177D was conducted over 2 × 10 min (1.5 mg cDNA/animal). At the same day, 10^7 fluorescent microspheres were injected into the left atrium. Reference blood samples were drawn from the arterial sheath at constant rate (4.1 ml/min). After extubation, L-NAME–treated animals received water containing 500 μmol/l of the NOS inhibitor for 3 weeks.

Hemodynamic measurements. At day 28 and day 49, global hemodynamic parameters (heart rate, left ventricular end-diastolic pressure [LVEDP], left ventricular pressure [LVP], contraction velocity [dP/dt_{max}], and relaxation velocity [dP/dt_{min}]) were obtained (Table 1). At day 49, analysis of regional myocardial function was performed assessing subendocardial segment shortening (SES) of ischemic versus nonischemic myocardium. Therefore, sonomi-

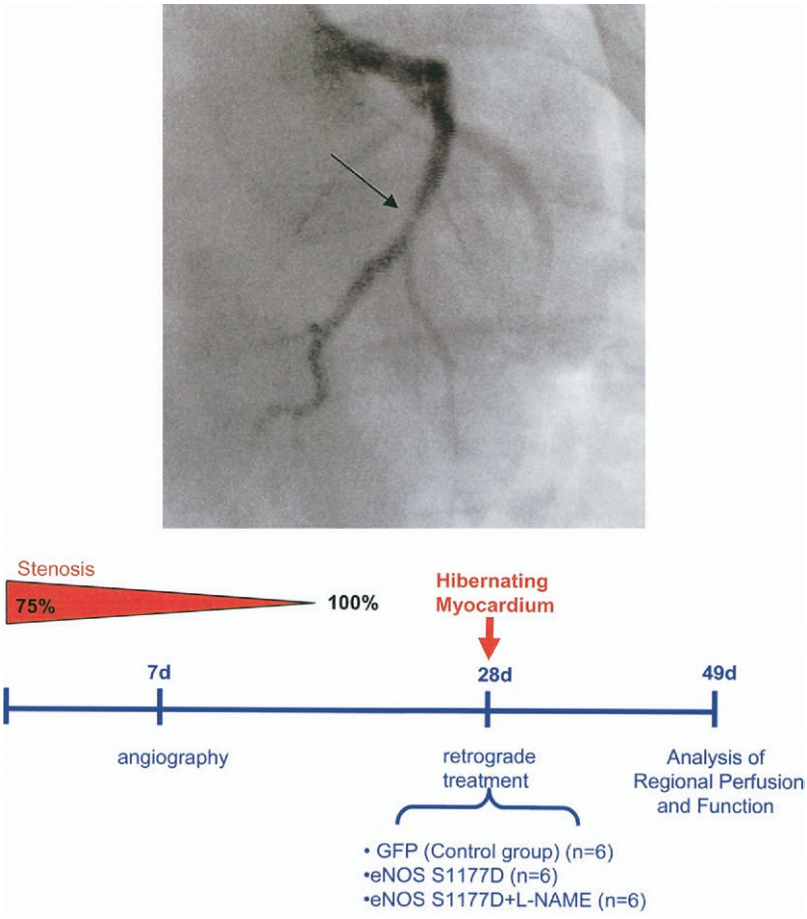

Figure 1 **Percutaneous Model of Hibernating Myocardium**

After percutaneous implantation of a reduction stent in the proximal left anterior descending artery (LAD) (day 0, **arrow**) the formation of a high-grade stenosis was angiographically confirmed at day 10 **(top)**. At day 28, (sub)total occlusion of the LAD was noted with distal filling of the LAD through collaterals. At this time point, retroinfusion of liposomes with either green fluorescent protein (GFP) or endothelial nitric oxide synthase (eNOS) S1177D was performed. At day 49, hemodynamic measurements were performed, and tissue was obtained for molecular analysis.

crometry crystal pairs were placed 1 cm (proximal LAD) and 3 cm (distal LAD) distal to the stent into the anterior LV wall. A pair of crystals were inserted in the circumflex coronary artery (Cx) perfusion area. The distance between each crystal pair was followed continuously and evaluated for 5 heart cycles at each heart rate (at rest, 120 beats/min, and 140 beats/min), as previously described (18). Results of the proximal and distal LAD-perfused area were normalized to the subendocardial segmental shortening of the control region (Cx perfusion area), given in percent.

Blood flow analysis, immunohistochemistry. For assessment of regional myocardial blood flow, fluorescence-labeled microspheres (molecular probes, 1×10^7) were injected into the left atrium at each time point (day 28, day 49). Reference blood samples were drawn from the arterial sheath. Systematic sampling of LV-myocardium (4 to 8 samples of each short axis slice of LV myocardium) allowed for quantification by an automatized fluorescence detection process (17). Perfusion levels at baseline and after rapid atrial pacing (140/min, flow reserve) were calculated as mean of the ischemic and (Cx-perfused) nonischemic area in the ischemic slices (slice 1 = ischemic region near basis, slice 4 = apex). Tissue from nonischemic and ischemic LV myocardium was used for immunohistochemistry with antibodies against eNOS and inducible NOS (iNOS) (Transduction Lab, Lexington, Kentucky), Ki67 (Novocastra), and PECAM-1 (Santa Cruz, California). For quantification, 5 fields were counted/region/heart, a microscopic high-power field containing 0.0875 mm^2. Immunoblotting was performed with tissue samples that had been frozen in a mixture of acetone and dry ice, on 12% sodium-dodecylsulfate (SDS)-polyacrylamide gels (Mini-Protean II, Bio-Rad, Hercules, California). After transfer to a polyvinylidene difluoride membrane (Bio-Rad), and blocking, membranes were incubated with a murine eNOS antibody (Transduction Lab) and subsequently with a goat anti-mouse immunoglobulin secondary antibody (Jackson ImmunoResearch Lab, West Grove, Pennsylvania).

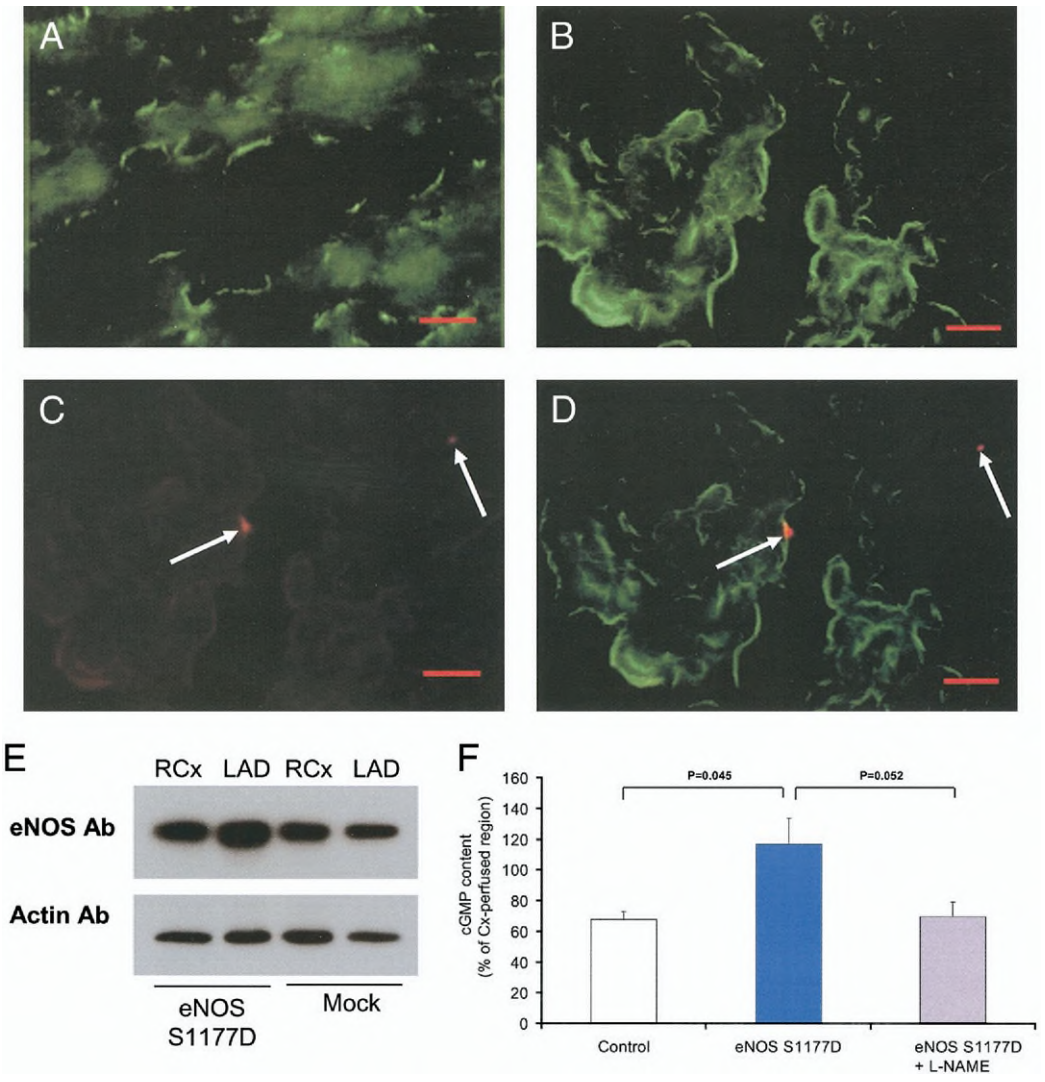

Figure 2 **eNOS Expression After Retroinfusion of eNOS S1177D**

Compared with a control heart **(A)**, endothelial nitric oxide synthase (eNOS) S1177D transfection enhanced eNOS expression in the ischemic area **(B)**. **(C)** In comparison, inducible NOS (iNOS) was found to be more confined to round cells **(arrows),** which did not express eNOS (**D** = merged fluorescence). **Red bar** = 50 μm. **(E)** Immunoblotting of eNOS revealed a 2.3-fold increase of eNOS expression in the ischemic region, as compared with the control region (representative example of 3 experiments). **(F)** Cyclic guanosine monophosphate (cGMP) levels detected by radioimmunoassay (RIA) indicate an increased cGMP formation in eNOS S1177D-treated animals, except for L-nitroarginine-methylester (L-NAME) co-application (normalized to nonischemic tissue, 3 animals/group with 3 tissue specimens/condition each). LAD = left anterior descending artery; RCx = ramus circumflexus.

Statistical methods. The results are given as mean ± SEM. Statistical analysis of results between experimental groups was performed with 1-way analysis of variance (ANOVA). Whenever a significant effect was obtained with ANOVA, we performed multiple comparison tests between the groups with the Bonferroni procedure. All procedures were performed with an SPSS statistical program (version 13.0, SPSS Inc., Chicago, Illinois). Differences between groups were considered significant for $p < 0.05$.

Results

eNOS overexpression and cell proliferation. In the ischemic area of transfected hearts, eNOS expression 72 h after transfection was found higher than in the nonischemic region, as depicted in Figure 2. Quantification of cyclic guanosine monophosphate (cGMP) content of the ischemic tissue (given in % of the nonischemic tissue of the Cx-perfusion area) revealed an increased formation of cGMP, a nitric oxide sensitive second messenger, in the eNOS S1177D treated hearts (Fig. 2F).

In vitro, eNOS transfection sufficed to induce increased proliferation of hypoxic coronary endothelial cells (Fig.3), an effect blunted by L-NAME co-application, indicating pro-angiogenic endothelial activation. In vivo, a consistently higher number of proliferating cell nuclei was detected in

Table 1 Hemodynamic Assessment of Pig Hearts

Experimental Group	Control Group	eNOS S1177D	eNOS S1177D + L-NAME
Heart rate (min^{-1})			
D28	88 ± 6	77 ± 4	84 ± 7
D56	85 ± 4	81 ± 5	85 ± 6
LVEDP (mm Hg)			
D28	18.7 ± 1.2	19.5 ± 0.6	18.4 ± 1.1
D56	20.5 ± 1.1	17.1 ± 0.8	20.6 ± 1.8
LVSP (mm Hg)			
D28	103 ± 4	100 ± 4	102 ± 3
D56	94 ± 4	96 ± 4	106 ± 5
dP/dt_{max} (mm Hg/s)	1,358 ± 111	1,334 ± 75	1,385 ± 24
dP/dt_{min} (mm Hg/s)	−1,195 ± 175	−1,122 ± 46	−1,316 ± 122

dP/dt_{max} = contraction velocity; dP/dt_{min} = relaxation velocity; eNOS = endothelial nitric oxide synthase; L-NAME = L-nitroarginine-methylester; LVEDP = left ventricular enddiastolic pressure; LVSP = left ventricular systolic pressure.

eNOS transfected myocardium (Figs. 4A to 4C). Co-staining with PECAM-1 (Fig. 4D) identified 42 ± 5% of the proliferating cells displaying Ki67 as endothelial cells, whereas 21 ± 2% displayed Ki67 and smooth muscle antigen. The L-NAME co-application with eNOS blunted endothelial cell proliferation (Fig. 4C).

Capillary density in hibernating myocardium. In vivo, improved perfusion relies on 2 components of the vasculature, microcirculatory vessels (i.e., capillaries and small arterioles) as well as conductance vessels such as collaterals. Three days after eNOS-transfection, capillary density of the ischemic area increased, compared with eNOS+L-NAME application (Fig. 5B). At day 21, capillary density after eNOS-transfect ion still displayed a trend to capillary growth (172 ± 13 capillary/field, p = 0.08), whereas after eNOS+L-NAME treatment capillary density was unchanged to control subjects (control: 145 ± 7; eNOS S117D + L-NAME: 155 ± 8 capillary/field).

Effect of eNOS S1177D on arteriogenesis. Collateral formation at day 28 after ischemia induction remained unchanged between day 28 (2.4 ± 0.4) and day 49 (1.9 ± 0.3) in the mock-transfected group. However, in the eNOS S1177D-transfected group the number of collaterals was significantly increased at day 49 (Fig. 5D). Again, concomitant L-NAME treatment prevented growth of this vessel segment.

Effect of eNOS S1177D transfection on myocardial blood flow. Because the structural prerequisites of effective neovascularization, conductance vessel, and microvessel growth have been detectable, the level of perfusion of hibernating myocardium before and after eNOS S1177D transfection was analyzed by injection of fluorescent microspheres. At day 28, coronary perfusion of the normoxic Cx-perfused myocardium did not differ between groups (Figs. 6A and 6B). At day 49, LAD perfusion of control animals was reduced at rest (62 ± 4% of Cx perfusion) (Fig. 6E) as well as at rapid atrial pacing (65 ± 3%) (Fig. 6F), while Cx-perfusion itself increased by 36% under pacing (data not shown). After eNOS S1177D-transfection, perfusion of ischemic area increased to 77 ± 3% (at rest) and 80 ± 5% (140/min) (Figs. 6C to 6F, Table 2). Addition of L-NAME prevented a significant increase of LAD-perfusion above control levels (69 ± 3% and 70 ± 5% of Cx-perfusion, respectively) (Figs. 6E and 6F).

Regional myocardial function at rest and atrial pacing. Given the chronic ischemic hibernating myocardium induced by a reduction stent, as used in the present study, changes in perfusion of the ischemic region were potentially capable of translating into changes of myocardial performance. We assessed regional myocardial function via sonomicrometry of the ischemic versus the normally perfused control area. Subendocardial segment shortening of the proximal ischemic LAD area amounted to 63 ± 12% of the Cx-perfused area and did not significantly change during atrial pacing or when compared with eNOS S1177D transfection with or without L-NAME. In constrast, in the distal LAD area, at higher heart rates an improved preservation of SES was found in eNOS S1177D-transfected animals (41 ± 8% and 33 ± 7% at 120/min and 140/min, respectively), whereas mock-transfected animals displayed a significant loss of myocardial function at 140/min (7 ± 4%) (Fig. 7). Regional contraction of the ischemic area after eNOS + L-NAME treatment did not differ from the level of mock-transfected animals.

Discussion

In the present study, we demonstrated that retroinfusion of eNOS S1177D cDNA (Fig. 2) into a region of hibernating myocardium was efficiently inducing neovascularization (Figs. 5 and 6) and improvement of myocardial function (Fig. 7) in a preclinical pig model. In vitro and in vivo analysis indicated a role of endothelial proliferation in the neovascularization response to eNOS S1177D overexpression (Figs. 3 and 4).

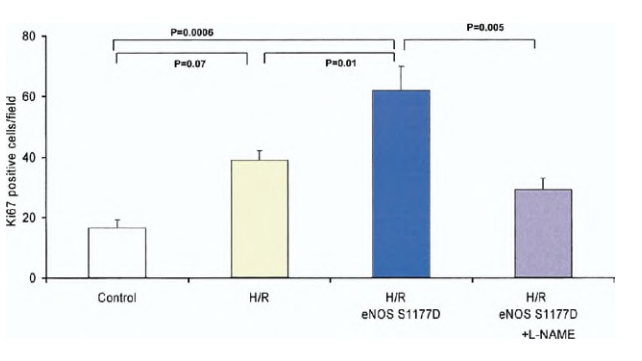

Figure 3 Endothelial Cells Proliferate After eNOS S1177D Transfection In Vitro

Proliferation of hypoxic coronary endothelial cells (CECs) is increased after endothelial nitric oxide synthase (eNOS) S1177D transfection (n = 4 independent experiments). H/R = hypoxia-reoxygenation; L-NAME = L-nitroarginine-methylester.

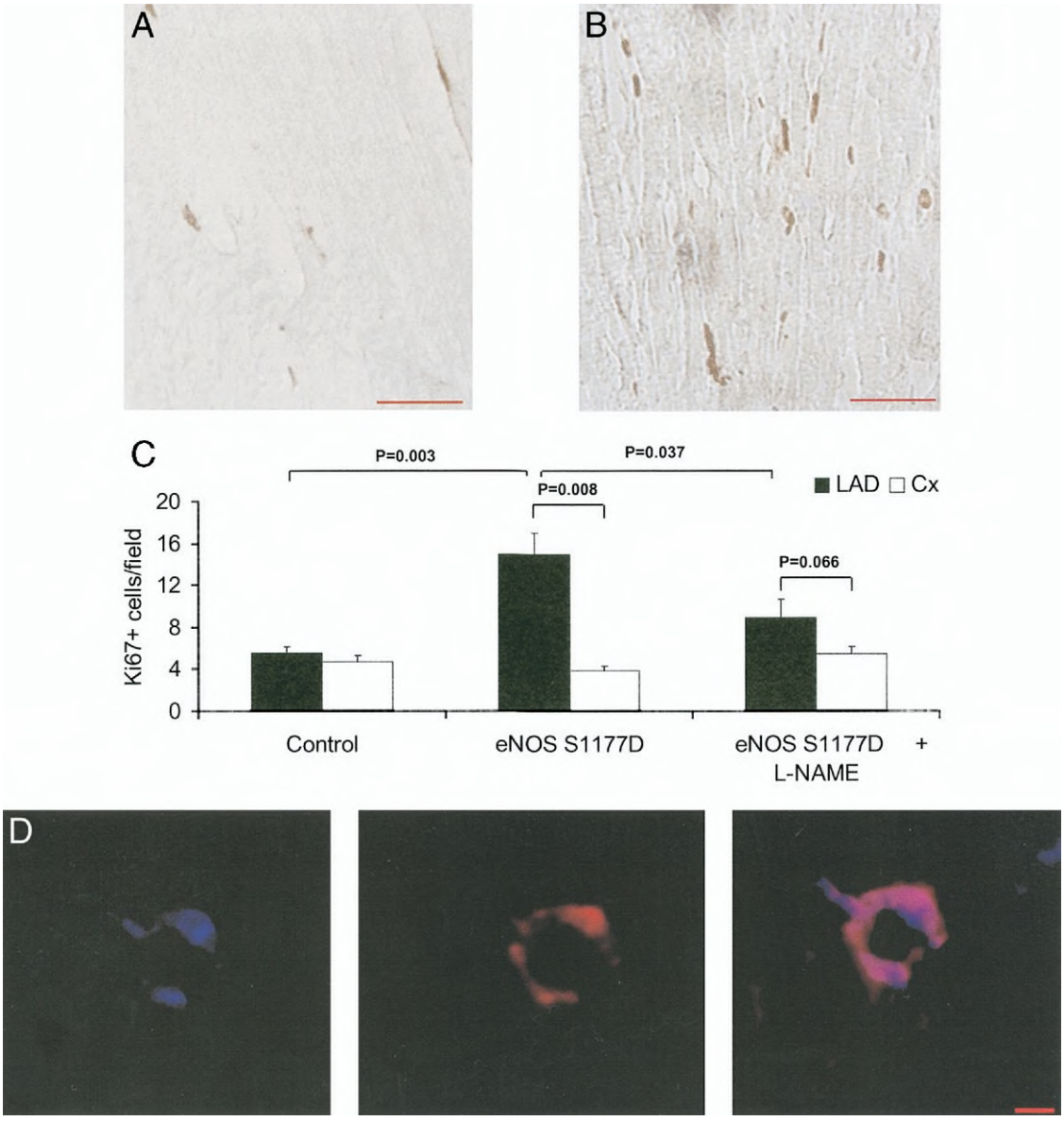

Figure 4 **Endothelial Cells Proliferate After eNOS S1177D Transfection In Vivo**

Histochemical evaluation proliferation (Ki67 positivity) in control **(A)** and ischemic **(B)** tissue. **Red bar** = 50 μm. **(C)** Quantitative analysis revealed an increased proliferation in eNOS S1177D transfected tissue (3 experiments/group with 5 tissue specimens/condition each). Cx = circumflex coronary artery. **(D)** Fluorescence microscopy of Ki67 **(left)**, endothelial marker platelet endothelial adhesion molecule-1 (PECAM-1) **(middle)**, and overlay **(right)**, **red bar** = 10 μm. Abbreviations as in Figure 2.

Model of hibernation. For induction of critical ischemia in the myocardium, we chose percutaneous reduction stent implantation, resulting in an acute 75% stenosis that progressed to total occlusion at day 28 (Fig. 1). Percutaneous implantation allowed for atraumatic induction of hibernation over 4 weeks, which was confirmed by ^{18} fluorodeoxyglucose (FDG)- and ^{13} ammonium (NH3)-positron emission tomography (17). Thereafter, myocardial perfusion of the ischemic region is unaltered in mock-transfected control subjects (Figs. 6C and 6D). Capillary densities at day 28 and day 49 indicate that rarification of microvessels is accompanying the development of hibernation (Fig. 5A), a phenomenon confirmed in patient biopsies (20). By virtue of the study protocol, treatment initiated at day 28 is exclusively targeting chronic ischemia, avoiding interference with the physiologic response to acute ischemia, which is also affected by eNOS (18). The gradual induction of ischemia by percutaneous reduction stent implantation is similar to ameroid constrictor ring placement, which requires, however, lateral thoracotomy with a larger wound area (21). This feature also accompanies another surgical approach, placement of a Delran occluder with a fixed internal

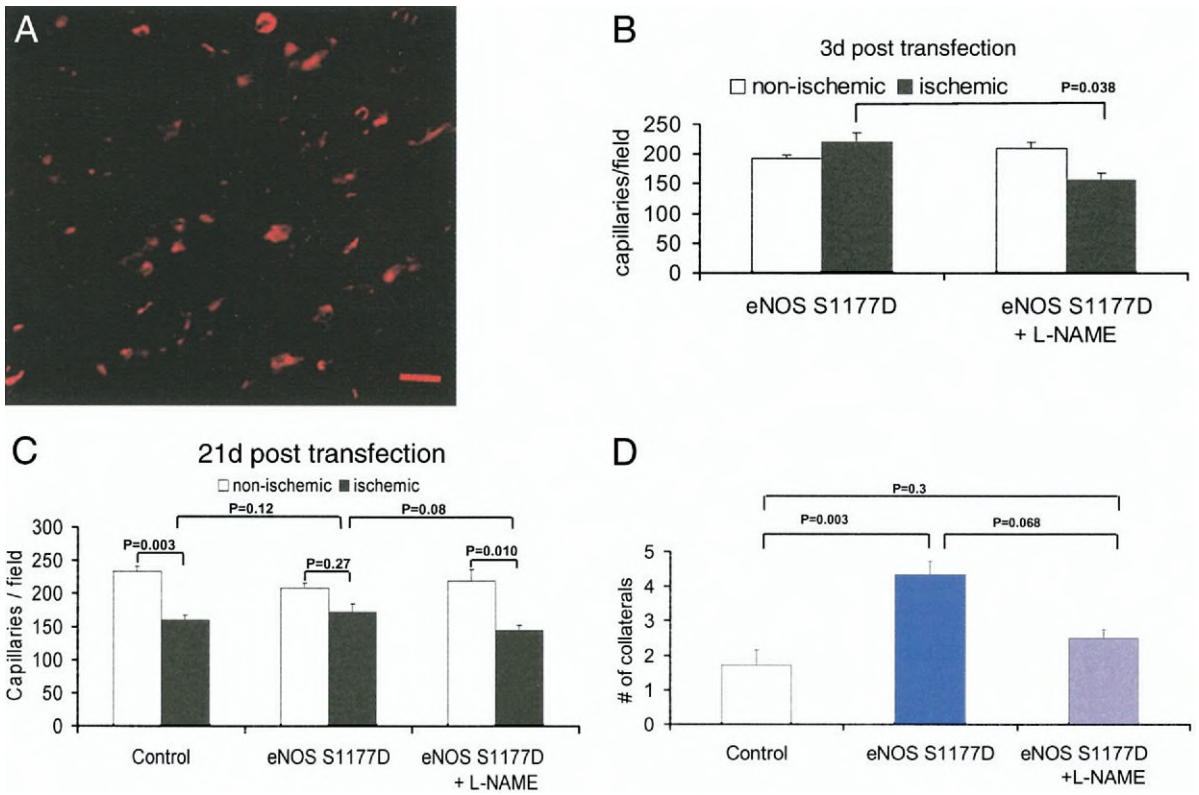

Figure 5 **Capillary and Collateral Growth After eNOS S1177D Transfection In Vivo**

(A) Example of platelet endothelial adhesion molecule-1 staining of capillaries (**red bar** = 100 μm). **(B)** Three days after transfection, the ischemic area of endothelial nitric oxide synthase (eNOS) S1177D-transfected animals displayed increased capillary levels compared with the eNOS+ L-nitroarginine-methylester (L-NAME) group. **(C)** The pro-angiogenic effect was found attenuated at day 49. **(D)** However, the number of collaterals at day 49 (postmortem angiogram) was increased after eNOS S1177D transfection (n = 6/group).

diameter around the target coronary vessel (22). In accordance with the latter model, basal flow in the ischemic myocardium of our experiments was decreased as compared with the nonischemic myocardium (Fig. 6), confirming hibernating myocardium.

eNOS expression. For eNOS S1177D transfection, retroinfusion of the liposome-cDNA mix was applied, as described in detail before (18). This application method particularly suits the context of chronic ischemia due to severe arterial stenosis, because the unaffected venous vasculature can be used for prolonged transfection applying flow reversal and concomitant venous outflow blockade. With that approach, a 2.3-fold overexpression of the eNOS construct is achieved in the ischemic region, in accordance with previous studies using this approach to transfect an ischemic myocardial region (18,23,24). This expression level sufficed to enhance the levels of cGMP—a second messenger formed upon nitric oxide exposure—in the ischemic tissue, unless the NOS inhibitor L-NAME was co-applied. Although L-NAME potentially exerts a detrimental effect by unselectively inhibiting NOS activity of native and forced NOS expression, we have not observed L-NAME–induced loss of function in pig hearts (18) at the dosage used in this study. Moreover, L-NAME uptake was stopped 24 h before the day 49 measurements in order to rule out an acute effect of NOS-inhibition.

eNOS and neovascularization. Although endothelial NOS is centrally involved in rapid regulation of vascular tone and vessel adhesivity, a role in vascular homeostasis has been suggested before (15,25–27). With respect to the elements of neovascularization, angiogenesis (25), arteriogenesis (28), and vasculogenesis (11), eNOS seems to play a significant role, in particular in VEGF-mediated vascular responses (12,26). Concerning vasculogenesis, regional overexpression of eNOS through a constitutively active mutant was unable to alter bone marrow mobilization (29). However, proliferation of endothelial cells was induced in vitro and in vivo (Figs. 3A and 4), increasing capillary density in the hibernating myocardial region (Fig. 5A). A similar observation has been obtained from studies in ischemic hindlimb models, where transfection (30) or activation of eNOS (31) resulted in increased capillary/muscle fiber ratios. Moreover, arteriogenesis, providing conductance vessels required for perfusion of distant tissue, was enhanced by eNOS transfection (Fig. 5D). Of note, monocyte recruitment, a hallmark of arteriogenesis, is not directly

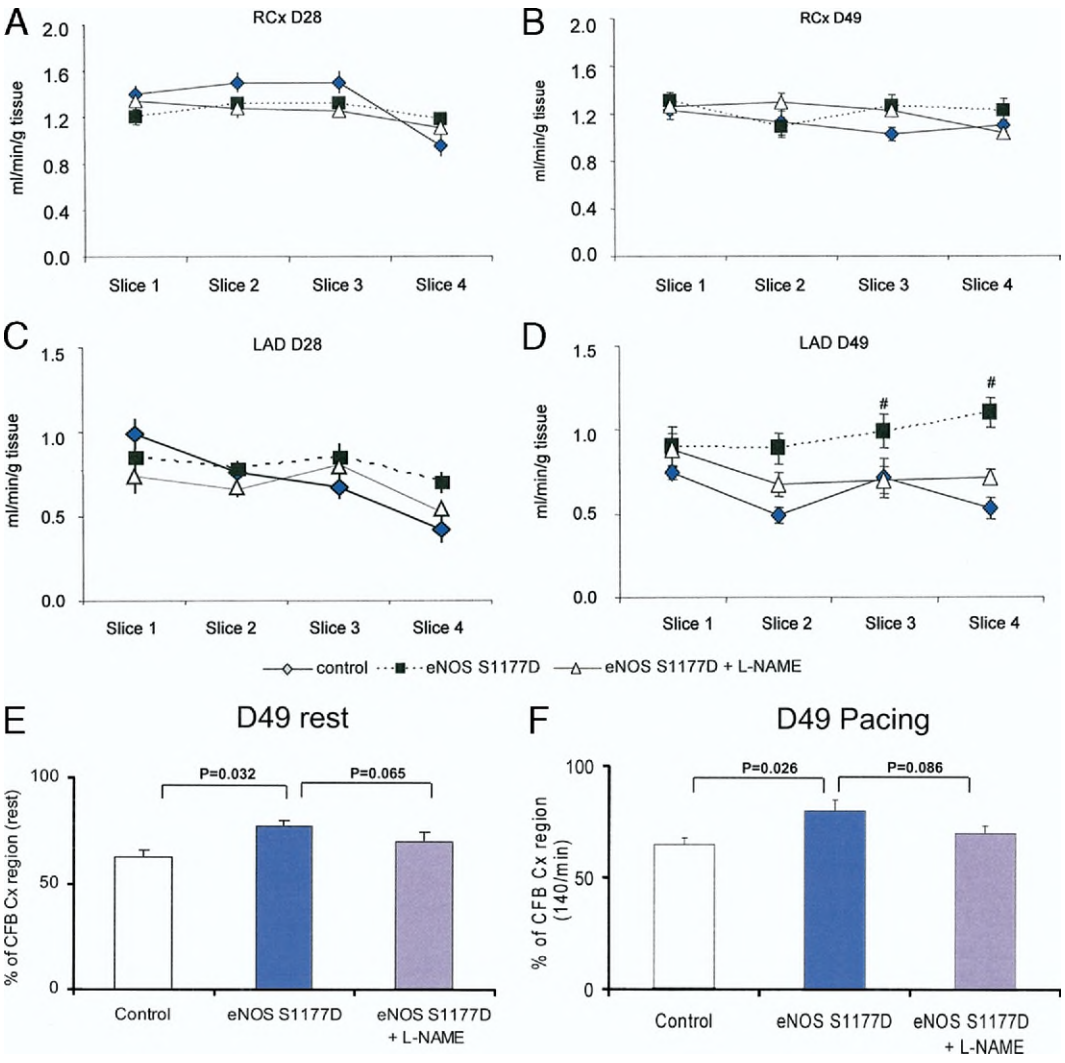

Figure 6 **Forced eNOS S1177D Expression Improves Perfusion of Ischemic Myocardium**

At day 28 **(A)** and day 49 **(B)**, regional myocardial perfusion in the RCx region at rest was similar in all groups at resting heart rate (slice 1 = most proximal ischemic short-axis tissue slice, slice 4 = apex). **(C)** The LAD perfusion at day 28 was reduced to the same extent in all groups. **(D)** Myocardial perfusion of the apical ischemic area was significantly improved in the eNOS S1177D-treated hearts, as compared with control or L-NAME co-treated animals. **(E)** At resting heart rate and **(F)** at rapid atrial pacing, the LAD perfusion/RCx perfusion ratio was significantly improved in the eNOS S1177D hearts, when compared with control hearts (n = 6 for control subjects, eNOS S1177D, n = 5 for eNOS S1177D + L-NAME). Cx = circumflex coronary artery; other abbreviations as in Figure 2.

upregulated by eNOS overexpression in vitro (data not shown). In contrast, monocyte chemoattractant protein (MCP)-1 application (32), which initiates arteriogenesis by enhancing monocyte recruitment, might induce vascular inflammation similar to an arteriosclerotic phenotype (33) and is associated with an unfavorable outcome in patients presenting with an acute coronary syndrome (34). This observation does not rule out a role of, for example, resident macrophages for arteriogenesis (35) after eNOS S1177D treatment but limits their triggering role for initiation of neovascularization induced by nitric oxide. In our study, a direct effect of nitric oxide formed in eNOS S1177D–transfected veins or increased shear stress due to a growing capillary bed (Fig. 5B)—which have also been observed in a chronic rabbit hindlimb ischemia model (31,36)—have most likely contributed to arteriogenesis after eNOS transfection. A similar combination of microvascular and macrovascular growth was achieved by chronic L-arginine supplementation in hypercholesterinemic pigs 7 weeks after ameroid ring placement (37).

Differential regulation of endothelial versus smooth muscle proliferation. In our experiments, immunohistology indicated that 1 hallmark of neovasculatory response of eNOS was cell proliferation. Co-staining identified the majority of proliferating cells displaying Ki67 positivity as endothelial cells, which are known to respond to eNOS activation by proliferation and migration (10,38). In contrast, L-NAME co-application with eNOS abolished endothelial cell prolifer-

Table 2 Regional Myocardial Blood Flow in Epicardial and Endocardial Tissue Samples

Experimental Group Perfusion (ml/min/g tissue)	Control Group		eNOS S1177D		eNOS S1177D + L-NAME	
	Epicardial	Endocardial	Epicardial	Endocardial	Epicardial	Endocardial
D28						
LAD	1.08 ± 0.15	1.08 ± 0.08	0.99 ± 0.07	1.01 ± 0.07	0.97 ± 0.11	0.99 ± 0.10
RCx	1.60 ± 0.04	1.62 ± 0.15	1.60 ± 0.12	1.57 ± 0.06	1.49 ± 0.14	1.55 ± 0.04
D56 rest						
LAD	0.85 ± 0.06	0.73 ± 0.05	1.09 ± 0.06*	1.06 ± 0.06*	0.94 ± 0.07	0.82 ± 0.06
RCx	1.38 ± 0.05	1.23 ± 0.06	1.39 ± 0.07	1.45 ± 0.05	1.37 ± 0.09	1.29 ± 0.06
D56 pacing						
LAD	1.19 ± 0.07	1.08 ± 0.06	1.62 ± 0.12*	1.41 ± 0.09*	1.45 ± 0.06	1.34 ± 0.09
RCx	1.77 ± 0.09	1.72 ± 0.04	1.97 ± 0.08	1.80 ± 0.07	2.02 ± 0.15	1.93 ± 0.11

*$p < 0.05$ versus corresponding tissue of the control group.
LAD = left anterior descending artery; RCx = ramus circumflexus; other abbreviations as in Table 1.

ation (Fig.3). Moreover, eNOS knockout mice display positive enhanced smooth muscle cell proliferation causing negative remodeling of a carotid artery after injury (28), whereas forced eNOS expression abolishes smooth muscle proliferation (39). These findings support the notion that eNOS activity controls neovascularization by reciprocally activating endothelial proliferation and inhibiting smooth muscle cell proliferation and luminal loss (28,29).

Clinical perspective. In summary, we report a neovasculatory response of chronic ischemic myocardium after regional transfection of a constitutively active eNOS construct (eNOS S1177D), which relied on capillary proliferation as well as collateral growth and was sensitive to NO-inhibition via L-NAME. Enhanced perfusion and an improved response to stress testing (rapid atrial pacing) indicate that this approach provided functionally relevant neovascularization. Although the sensitivity of experimental detection of perfusion and function of the ischemic myocardium might not be directly translated to the clinical setting, recent advances in magnetic resonance imaging for clinical assessment of coronary perfusion (40,41) and function (42) seem particularly promising for detection of perfusion changes induced by neovascularization. Moderately enhancing NO bioavailability via eNOS cDNA retroinfusion might offer a therapeutic approach in patients with hibernating myocardium not accessible to conventional revascularization.

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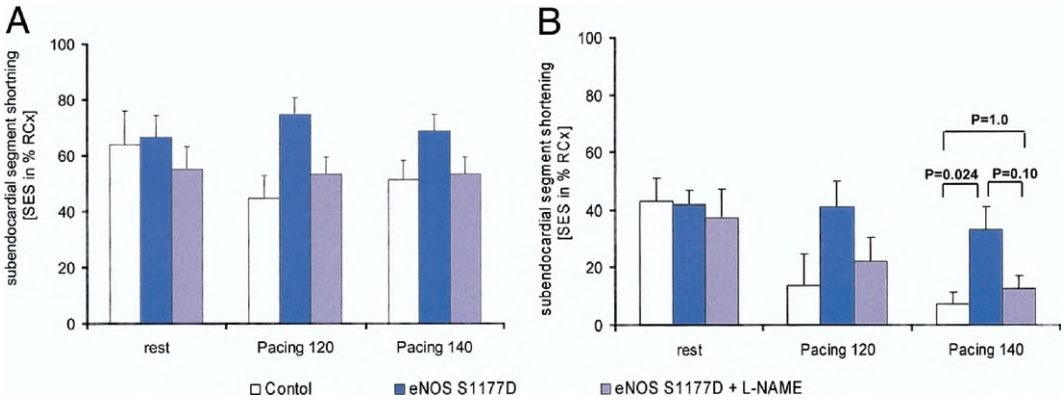

Figure 7 Regional Gain of Myocardial Function in the Distal LAD Region

Regional myocardial function was assessed by subendocardial segment shortening (see Methods) at day 49 in the proximal **(A)** or distal LAD region **(B)** at rest or after atrial pacing (120/min and 140/min); n = 5/group. Abbreviations as in Figure 2.

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